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(54) Title: THE PRODUCTION OF SOLUBLE KERATIN DERIVATIVES

(57) Abstract: A process for the preparation of soluble proteins of high molecular weight with little or no damage to the structural integrity of the proteins. The process is economically and environmentally acceptable by virtue of the cost of reagents that are used, and the recycling of some of those reagents, and is suitable for the production of soluble proteins on a large scale. The process includes a first stage using oxidative sulfitolysis followed by a second stage using mild conditions to extract the soluble protein. In the case of wool as the protein source the process leads to the production of soluble keratin proteins fractionated into the classes S-sulfonated keratin intermediate filament proteins and S-sulfontated keratin high sulfur proteins.

The Production of Soluble Keratin Derivatives

Field of the Invention

This invention relates to a process for the preparation of derivatives of keratin from animal sources such as wool, hair, horns, hooves, feathers and scales by an economic and environmentally acceptable process, and to a series of keratin derivative products produced thereby. Some of the keratin derivatives are soluble and can be used in the production of a range of biopolymer materials.

Background to the Invention

Keratins are a class of structural proteins widely represented in biological structures, especially in epithelial tissues of higher vertebrates. Keratins may be divided into two major classes, the soft keratins (occurring in skin and a few other tissues) and the hard keratins, forming the material of nails, claws, hair, horn, feathers and scales.

The toughness and insolubility of hard keratins, which allow them to perform a fundamental structural role in many biological systems, are also desirable characteristics in many of the industrial and consumer materials currently derived from synthetic polymers. In addition to possessing excellent physical properties, keratin, as a protein, is a material with a high degree of chemical functionality and, consequently, exhibits many properties that synthetic materials cannot achieve. Keratin is, therefore, well suited to the development of products with high-value, niche-market applications. Keratin is also an environmentally acceptable polymer produced from a sustainable resource and therefore has environmental benefits

over synthetic materials. Following the global trend of developing materials from renewable sources produced in a sustainable process, a range of materials has been produced from keratin, most commonly in the form of keratin films.

At the core of a new industry producing biopolymer materials from keratin it is essential to have a process for extracting keratin from its source that is economically viable, sustainable from an environmental perspective, and produces a stable and versatile product. Methods used to date for the extraction of keratin that maintain the integrity of the individual proteins have been designed for the purpose of protein analysis and characterisation and consequently are not viable on an industrial scale, from an economic and environmental viewpoint. Methods used to date for the economic dissolution of keratin have significantly degrading effects on the protein, and consequently the dissolved protein retains few of the physicochemical properties that lead to the desirability of keratin as a biopolymer, such as the ability to reconstitute into tough materials.

It is an object of the invention to go some way in overcoming the disadvantages with known processes or at least provide the public with a useful choice.

In at least one embodiment the invention strives to provide an economic and environmentally acceptable process for the dissolution of keratin proteins that maintains the structural integrity and chemical functionality of the proteins during the dissolution process and leads to a stable and versatile keratin derivative product for the development of biopolymer materials.

Summary of the Invention

According to a first aspect the invention provides a dissolution process for producing a range of stable, soluble keratin derivatives of high molecular weight, the molecular weight being similar to or greater than that of proteins originally expressed in the keratin source, with little or no damage to the structural integrity of the constituent proteins. The dissolution occurs in a two-stage process.

According to a preferred aspect the invention provides a process for the preparation of keratin derivatives of high molecular weight, the process including a first stage digestion step of S-sulfonating a keratin source by oxidative sulfitolysis followed by a second stage extraction step using controlled washing with water to thereby obtain a highly S-sulfonated keratin derivative.

The conversion of highly S-sulfonated keratin from a solid state into solution is without the use of chaotropic agents, by controlled, gradual washing of the sulfonated keratin with water in order to wash out the residual chemical reagents from the extraction procedure and alter the ionic strength of the extraction solution.

The first stage involves oxidative sulfitolysis to convert cystine groups present in the protein to S-sulfocysteine, using industrially acceptable concentrations of inexpensive reagents for the purpose of sulfonation (eg. sodium sulfite) and oxidation (eg. cupraammonium hydroxide).

According to another aspect, the invention provides a process for the separation of a gelatinous keratin product from a solution of S-sulfonated keratin produced by

the above process, wherein the S-sulfonated keratin derivative solution is treated by the use of a gentle, gravity fed filtration system followed by separation. Preferably the separation is centrifugal.

According to another aspect of the invention, a liquid stream remaining after the gelatinous keratin is removed is processed by passing over scoured wool, thereby removing residual chemicals from the solution and preparing the wool for subsequent protein extraction processes.

Following conversion of the cystine groups, the second stage of the process is one in which the highly S-sulfonted keratin derivative is brought from a solid or gelatinous state into solution by extensive dilution with water. The rate and extent of dissolution can be controlled by the use of heat, surfactants, gentle agitation and vigorous chopping or homogenisation. By controlling the rate of dissolution, reaction solutions can be isolated, for example if a copper oxidant is used a reaction solution rich in copper is produced but it contains little or no dissolved protein, or are rich in protein but contain little or no copper.

According to another aspect of the invention, a liquid stream resulting from the second stage of the process, which contains residual chemicals such as copper sulfate and sulfite, as well as S-sulfonated keratin derivatives, is processed using any one or more of a variety of methods that allow the recycling of reagents from the solution and the separate isolation of purified S-sulfonated Keratin Intermediate Filament Protein(s) (SIFP) and S-sulfonated Keratin High Sulfur Protein(s) (SHSP). This is achieved through the use of chelating agents, such as ethylenediamine

tetraacetic acid, or chelating ion exchange resins, such as those containing the iminodiacetic functional group, and the use of isoelectric precipitation to separate protein types. Ultrafiltration can be used at several stages in the process to improve the efficiency of reagent removal or protein separation. Metallic impurities in the protein products can be further reduced by the washing of the protein derivative(s) following precipitation with dilute acids, water or chelating agents. Once separated, the protein products can be dried by a range of methods such as fluid bed, spray or freeze drying.

Another aspect of the invention is the further processing of residual keratin not dissolved by the two stage sulfitolysis process, through the use of other reagents, such as hydrogen peroxide, sodium sulfide or proteolytic enzymes, to produce keratin peptides.

Another aspect of the invention is the provision of a method for large scale recovery of proteins from a natural source, including subjecting said natural protein source to a treatment sufficient to render at least some of the protein(s) water soluble, and subsequently separating the water soluble protein(s).

Another aspect of the invention is the provision of an installation for large scale recovery of proteins from a natural source, a treatment vessel to contain and subject a large quantity of natural protein source to a treatment sufficient to render at l ast some of the protein(s) contained in said feed, water soluble, and a separation apparatus to subsequently separate the water soluble protein(s).

Another aspect of the invention is a method of selectively solubilising a protein having plurality of disulfide bonds from a mixture of proteins including subjecting said mixture of proteins to oxidative sulfitolysis to produce a soluble S-sulfonated protein fraction. The oxidative sulfitolysis is preferably effected in the absence of chaotropic agents with little or no damage to the structural integrity of the protein.

Another aspect of the invention is method for obtaining a purified protein from an impure protein source with little or no damage to the structural integrity of the protein including subjecting said protein source to a treatment sufficient to render at least some of the protein(s) water soluble, and subsequently separating the water soluble protein(s) in the absence of chaotropic agents.

Description of Preferred Examples of the Invention

The combination of aspects that make up the process as a whole are summarized diagrammatically in attached Figure 1.

This process method is for the preparation of highly sulfonated keratin derivatives and can be applied to any keratin source, such as animal wool, hair, horns, hooves, feathers or scales. Whilst the application of the method to different keratin sources can give soluble keratins with different structure and properties, the fundamental step of the dissolution process, in which cystine is converted to s-sulfocysteine, applies equally well to all keratin-containing materials.

The process can be conceived as occurring in two stages.

Stage one, which involves the conversion of cystine to S-sulfocysteine, occurs through a procedure of oxidative sulfitolysis. This can be achieved by the use of a sulfonating agent, such as sodium sulfite or sodium metabisulfite, which asymmetrically cleaves the cystine to cysteine and S-sulfocysteine, and an oxidant, which converts the cysteine produced in sulfonation to cystine. By further sulfonation of cystine complete conversion of all cystine to S-sulfocysteine is achieved.

Oxidants which can be used include sodium tetrathionate, iodosobenzoate and cuprammonium hydroxide. In a preferred embodiment of this invention the sulfonating reagent used is sodium sulfite in the concentration range 0.02M to 0.2M and the oxidant used is cuprammonium hydroxide in the concentration range 0.02M to 0.08M, generated by the combination of copper sulfate and ammonia. The first stage of the procedure for solublising keratin is the soaking, for a residence time such as 24 hours, of the keratin source in a solution or sequence of solutions that convert the cystine to S-sulfocysteine, with a liquor to wool ratio (volume:weight) in the range 5:1 to 50:1.

In another embodiment of the invention the sulfonating agent used is sodium metabisulfite in the concentration range 0.1M to 0.5M, maintained at acidic pH. In this embodiment the wool is removed from the solution containing sodium metabisulfite before being added to a solution containing a cuprammonium complex in the concentration range 0.02M to 0.08M.

Previous work relating to the use of the oxidative sulfitolysis procedure has required the use of large concentrations of chaotropic agents, such as urea or guanidinium hydrochloride, in order to swell the keratin source and facilitate the dissolution of keratin. This procedure is both expensive and impractical on an industrial scale. Previous work relating to the use of oxidative sulfitolysis using copper as the oxidant has been conducted under conditions of temperature and pH that are detrimental to the integrity of the protein causing high rates of conversion of cystine to lanthionine.

Stage two of the process involves the conversion of highly sulfonated keratin from a solid state into solution without the use of chaotropic agents and under conditions of temperature and pH that maintain the structural integrity of the protein, by controlled, gradual washing of the sulfonated keratin with water in order to wash out the residual chemical reagents from the extraction procedure and alter the ionic strength of the extraction solution. This combination of effects results in the conversion of the highly sulfonated keratin from the solid state into aqueous solution. In the preferred procedure the reaction volume is replaced every 12 to 48 hours, either in a batch process or on a continuous basis.

The rate and extent of dissolution can be controlled by the use of surfactants, the action of heat, agitation, and homogenisation of the sulfonated keratin. A feature of the invention is to use these factors to control the rate of extraction. The highly S-sulfonated keratin can, therefore, be kept in the solid state and separated from the extraction solution containing the bulk of the chemicals used for the

sulfonation process. The preferred procedure uses a non-ionic surfactant, such as Triton X 100 in the range 0.1% to 5% by weight, and a temperature maintained in the range 15°C to 50°C.

An advantage of the invention when a copper based oxidant is used is the re-use of this copper-rich extraction solution for subsequent extraction processes, significantly reducing both the cost and environmental impact of the process. Re-use of the copper-rich solution is possible due, in part, to the regeneration of the active copper species through aerial oxidation. One method in which the copper-rich solution can be efficiently reused is by passing the solution over wool. Wool binds copper from the solution, and if this wool is then used for subsequent extraction processes, the demand for copper in those subsequent extractions is reduced. In this way, a 'wool filter' can be used as a key step in the processing of the copper-rich extraction solution, reducing the subsequent need for effluent treatment and also the need for copper to be added to the subsequent processes. In a typical procedure the liquid stream from stage 1 contained approximately 1800 – 1500 (parts per million) ppm copper, and after passing over the wool filter this was reduced to approximately 400 - 300ppm.

The first stage of the process, and the recovery of reagents for use in the process are indicated in the attached Figure 1.

After S-sulfonation and homogenisation the keratin material becomes a gelatinous swollen fibrous mass.

A further advantage of the invention is the separation of the highly S-sulfonated keratin derivatives in the solid state from solutions containing either chemicals used in the extraction process or the keratin protein in solution. This separation is effectively achieved by the use of a gentle, gravity based filtration through a fine mesh screen, followed by centrifugal separation of the filtrate from fine particulates.

Solutions of highly S-sulfonated keratin derivatives can be purified with regard to metal ions, specifically the copper ions used as part of the extraction process, through the use of ion exchange media, in particular those containing iminodiacetic acid functionality known to possess a high affinity for divalent metal ions. This ion exchange medium may be present in the form of a packed resin column, over which the protein solution is passed, or it may alternatively form part of an electrochemical cell, in which copper is recovered from the ion exchange medium through the use of an applied voltage and a system containing permeable membranes.

Once the highly S-sulfonated keratin derivates are in solution particular proteins eg. the S-sulfonated keratin intermediate filament protein can be readily isolated by isoelectric precipitation, around pH 4 or below, using acids such as sulfuric acid, hydrochloric acid, citric acid or acetic acid, with the preferred procedure using sulfuric acid. An advantage of the invention is the minimisation of the binding of copper and other metallic impurities to the protein prior to isoelectric precipitation through the use of ion exchange media as described, or by addition of a chelating agent, such as ethylen diaminetetraacetic acid (EDTA), to the protein solution. In

the preferred example EDTA (0.2M) is added to the liquid stream from stage 2 at a rate of 25ml per liter, or at a rate suitable to sequester all the copper ions present in solution as indicated by analysis of the solution. Metallic impurities can be further reduced by the washing of the protein, once isolated by precipitation, with a dilute acid solution, or solution of a chelating agent such as EDTA, or water.

Following precipitation and washing the separated protein can be isolated in a stable, dry state using drying methods involving air flows at about ambient temperature, for example with the use of a fluid bed dryer. Alternatively, the product can be dried using a freeze dryer. The dry protein product contains cystine groups in the form of S-sulfonic acid and consequently the protein is only soluble in the presence of a base, such as sodium hydroxide or ammonium hydroxide. These processes are represented as drying in the attached Figure 1.

The highly soluble keratin derivatives that remain in solution following isoelectric precipitation, which in the case of wool are mainly the high sulfur matrix proteins from within the wool fibre, can be isolated in a stable form from solution through a process of ultrafiltration, to remove non-proteinacious species such as residual copper or EDTA, followed by spray drying.

A feature of the invention is the use of a combination of isoelectric precipitation and ultrafiltration followed by spray drying to separate highly S-sulfonated keratins according to their properties in solution. In the case of wool keratin, this effectively separates the low sulfur intermediate filament protein class from the

high sulfur matrix protein class and provides two product streams with different chemical properties.

A feature of the invention is the preparation of a stable, water soluble form of the highly S-sulfonated keratin derivative, by dissolving the S-sulfonic acid form of keratin in the presence of base and spray drying the resulting solution.

A feature of the invention is the combination of engineering components to allow solublisation of the keratin and isolation of the S-sulfonated keratin from solution in a continuous, semi-continuous, or batch process. This combination of engineering components and unit operations is detailed in Figure 1.

An advantage of the invention is the recovery and reuse of copper from the reaction mixtures and effluent streams of the process. Copper can be recovered using electrochemical methods, including the use of selective permeable membranes in order to separate copper ions from EDTA prior to electrochemical deposition. Alternatively, immobilized binding agents, in the form of copper-specific ion exchange resins, can be used to remove copper from the effluent stream. Copper removed using these methods can be reused, thereby minimizing the environmental impact of the process.

The use of ion exchange media and/or chelating agents is represented as purification in the attached Figure 1.

Another advantage of the invention is the further processing of residual keratin which remains in the solid state following the extraction procedure. This functionalised keratin is highly S-sulfonated, therefore the disulfide bonds present in the native keratin that render it resistant to chemical and enzymatic attack have been cleaved and the keratin is readily digestible using other extraction methods. For example, a solution rich in keratin peptides can be prepared through the action on this residual keratin of alkaline solutions of a strong oxidant such as hydrogen peroxide, in the concentration range 10-100ml of 50% hydrogen peroxide per kg of the keratin residue under alkaline conditions. The keratin residue contains approximately 5% solids. Alternatively, solutions of strong reductants such as sodium sulphide in the concentration range 0.5%-15% added to the keratin residue can be used to prepare a solution rich in keratin peptides. Alternatively, proteolytic enzymes, such as those of the subtilisin, papain or trypsin groups, can be employed at levels in the range 0.1mg – 20mg of enzyme per gram of keratin residue at temperature and pH conditions appropriate for the specific enzyme to readily digest the residual keratin and prepare a solution rich in keratin peptides. All of these methods result in the formation of a solution rich in keratin peptides which can be processed in a similar manner to the liquid stream resulting from stage 2 described above, that is through the use of ion exchange media, pH adjustment and drying (shown as purification and drying in Figure 1), to produce keratin peptide solids. Digestion of the keratin residue in this way minimises the keratin waste produced by the process as a whole, and ensures maximum utility of the keratin protein present in the keratin source.

The two intact protein products from the process are S-sulfonated keratin intermediate filament protein and S-sulfonated keratin high sulfur protein. The S-sulfonated keratin intermediate filament protein typically produced by the process was analysed using sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) analysis using a reduction/alkylation procedure, which indicated a molecular weight distribution predominantly in the range 30-60kD (intermediate filament proteins), with a small component of protein of mass 10kD (high glycine high tyrosine proteins). The amino acid composition of this product is given in Table 1 and is typical for wool keratin intermediate filament proteins. The S-sulfonated keratin high sulfur protein was analysed using SDS-PAGE after a reduction/alkylation procedure, which indicated a molecular weight predominantly in the range 15-20kD. The amino acid composition of this product is given in Table 1 and is typical for wool keratin high sulfur proteins.

	Cya	Asp	Glu	Ser	Gly	His	Arg	Thr	Ala	Pro	Tyr	Val	Met	Lan	Ile	Leu	Phe	Lys	Cys
SIFP	0.4	7.9	15.4	10.9	8.1	0.9	7.9	6.5	7.5	5.4	1.1	6.5	0.2	0.2	3.7	8.9	2.5	2.1	4.2
SHSP	1.7	2.6	8.6	14.3	9.1	0.8	6.8	10.4	3.6	12.6	1.8	6.3	0.0	0.2	2.9	3.9	1.5	0.4	12.4
IFP	0.0	9.6	16.9	8.1	5.2	0.6	7.9	4.8	7.7	3.3	2.7	6.4	0.6	0.0	3.8	10.2	2.0	4.1	6.0
HSP	0.0	2.3	7.9	13.2	6.2	0.7	6.2	10.2	2.9	12.6	2.1	5.3	0.0	0.0	2.6	3.4	1.6	0.6	22.1

Table 1: amino acid composition of S-sulfonated keratin intermediate filament protein (SIFP), S-sulfonated keratin high sulfur protein (SHSP), intermediate filament protein (IFP) and high sulfur protein (HSP) (later two courtesy of *Gillespie and Marshall, Variability in the proteins of wool and hair, Proc. Sixth Int. Wool Text. Res. Conf., Pretoria, 2, 67-77, 1980*). All residues expressed as mol%. S-sulfocysteine, cystine and cysteine are measured as S-carboxymethyl cysteine following reduction and alkylation.

An example of the process is shown diagrammatically in the attached Figure 1. Ultrafiltration is considered as being a possible component in each purification stage. The key components are illustrated by the following examples of a protein extraction procedure.

Examples:

Example 1 Stage 1, Digestion

The digestion stage of the process involves the use of oxidative sulfitolysis to convert cystine to S-sulfocysteine within the keratin source.

Example 1a. stage 1, digestion

In order to extract the keratin from 10kg of wool, firstly 2kg of copper sulfate pentahydrate was mixed using a high shear mixer with eight litres of concentrated ammonia. This mixture was diluted to 200L with water and 10kg of wool was added. Approximately 15L of sulfuric acid (2M) was added to the stirred mixture till a pH 9.4 was achieved. Anhydrous sodium sulfite (5.04kg) was added and the solution mixed until complete dissolution of all of the reagents had occurred and the pH stabilised at 9.5. The final concentration of the cupric ammonia complex was 0.04M. The sodium sulfite had a final concentration of 0.2M. The temperature of the digestion solution was maintained at 20°C. After 24 hours of gentle agitation the fibrous gelatinous mass of softened wool was filtered. The filtrate was passed through a fresh wool filter, which decreased the copper level in the solution from 1725ppm to 130ppm, and further purified using Purolite S930 IDA ion exchange resin, which under acidic conditions further reduced the copper level to 12ppm. Fresh water was added to the softened wool and the mixture was agitated.

Example 1b. stage 1, digestion with the use of surfactant.

In a variation of example 1a, the digestion solution was prepared with the addition of 1% of a non-ionic surfactant Triton X 100. The addition of this surfactant resulted in a delay in the release of soluble protein from the fibre, allowing a more

effective separation of protein from residual reagents such as copper salts in the extraction solution.

Example 1c. stage 1, digestion.

In a variation of example 1a, the digestion stage occurs in two parts. In the first part wool is pretreated with sodium metabisulfite at a concentration of 0.2M, at pH 4.2. Following removal of the wool from this solution, and with no attempt to remove residual sulfite from the wool, the wool was immersed in a cuprammonium hydroxide solution, at the concentration and pH described in example 1a for a further 24 hours at 20°C.

Example 2. Stage 2, Extraction.

Example 2a. stage 2, batch extraction.

Following completion of stage 1, described in examples 1, the mixture was agitated for a period of 16 hours, before being homogenized. Following a further 4 hours of agitation the solids and solution were separated using a two-stage filtration process involving a wedge wire screen followed by a settling tank and a spinning disc centrifuge. The solid phases were returned to the reaction vessel and water was added to give a final liquor to wool ratio of 20:1 based on original wool solids. Following 24 hours agitation or continuous homogenisation the mixture was separated by repeating the two-stage filtration process. The solid phases were returned to the extraction vessel and further diluted. This cycle was repeated 7 to 12 times. The liquid phases, containing soluble proteins, were further processed as detailed below in example 3.

Example 2b. stage 2, continu us extraction.

Following completion of stage 1 the mixture was processed as described in example 2a, except that the two stage filtration process occurred on a continuous

process, and solids and fresh water were added to the reaction tank at a rate equivalent to the volume of the tank being replaced in 24 hours. This process was continued for 120 hours.

Example 3. Processing of protein solutions.

Ultrafiltration can be used at several points during the processing of protein solutions, in order to concentrate solutions and make the processes of drying and ion exchange more efficient. Ultrafiltration may be used prior to any processing step outlined in the following examples.

Example 3a. processing of protein solutions using EDTA

The solution produced as a result of stage 2, as described in Example 2, was further processed to isolate purified soluble keratins. EDTA (0.2M) was added to the liquid phase at a rate of 25mL per liter, or at a rate suitable to sequester all the copper ions present in solution as indicated by analysis of the solution. Following 1 hour of mixing, the pH of the filtrate was reduced to 3.5 using sulfuric acid.

The protein precipitate was isolated using a screen, and washed sequentially with dilute sulfuric acid and water. The protein, S-sulfonated keratin intermediate filament protein, was dried by one of three routes, freeze drying, fluid bed drying or spray drying following dissolution with dilute sodium hydroxide. The filtrate following the protein precipitation procedure was further processed using ultrafitration, to separate the protein components from the residual reagents. The retentate was spray dried to isolate further soluble protein, S-sulfonated keratin high sulfur protein. The permeate was further processed to recover copper and EDTA from the effluent stream using ion exchange media.

Example 3b. processing of pr tein solution using ion exchange media

The solution produced as a result of stage 2, as described in Example 2, was further processed to isolate purified soluble keratins. The liquid phase was passed over ion exchange resin, such as the chelating resin Purolite S930 IDA ion exchange resin containing the iminodiacetic acid functional group, in order to remove copper ions from the solution. Following ion exchange the pH of the filtrate was reduced to 3.5 using sulfuric acid and further processed in an identical manner to that described for Example 3a.

3c. processing of protein solution using pH adjustment prior to ion exchange.

The solution produced as a result of stage 2, as described in example 2, was further processed to isolate purified soluble keratins. The pH of the liquid phase was reduced to 3.5 using sulfuric acid. The protein precipitate was isolated using a screen, redissolved using dilute sodium hydroxide and further purified with either the addition of EDTA or by passing over an ion exchange column. Following further purification, the pH of the solution was reduced to 3.5 using sulfuric acid and the protein was isolated as described in the earlier examples. The filtrate from the initial pH reduction step, which still contains significant amounts of soluble protein and other reagents, was purified by passing over ion exchange media and spray dried to isolate further soluble protein, S-sulfonated keratin high sulfur protein.

Example 4. Dissolution of residues from stage 2

The solid stream isolated as a result of stage 2 can be further processed to produce keratin peptides by a range of methods. The high level of sulfonation of the residue makes it readily amenable to chemical and enzymatic digestion, as the disulfide bonds present in the original keratin source resistive to chemical and enzymatic attack have largely been cleaved.

Example 4a dissolution of residues using sodium sulfide

Sodium sulfide solution (5% by weight) is added to an equal volume of the solid stream from stage 2 of the process, which comprises approximately 5 % solids. The mixture is agitated for 12 hours after which time the solids are removed by filtering and centrifugation and sulfuric acid is added to the protein solution to decrease the pH to the range 2 to 3.5. The precipitate is collected on a screen and washed thoroughly with water.

Example 4b dissolution of residues using hydrogen peroxide

Hydrogen peroxide (50%) is added to the solid stream from stage 2 at a level of 25–30 ml per kg of keratin residue (keratin residue contains approximately 5% solids). This is mixed and 1M sodium hydroxide is added to obtain pH in the range of 10 to 13. The mixture is agitated gently for 24 hours and the protein and solids separated by the two stage filtration process described in Example 2 and protein isolated by acidification as described in Example 4a. Alternatively the protein solution is passed over an ion exchange resin, then acidified and the precipitated solid collected. The acidified solution may then be passed through an ion exchange column prior to freeze-drying or spray drying to collect a further protein-rich product.

4c dissolution of residues using proteolytic enzymes

An industrial subtilisin enzyme preparation (a solution containing 2.5% active enzyme) was added to the solid stream from stage 2 in the amount of 10mg of active enzyme per gram of keratin residue. The pH was maintained at 9.5 with the addition of sodium hydroxide and the reaction heated to 60 °C for 2 hours. The resulting protein solution is isolated from solids and processed as described in 4a

or passed through ion-exchange resin prior to and/or following acidification as described in 4b.

Thus by the invention there is provided a method for the production of soluble keratin derivatives that is both economic and environmentally acceptable.

Particular examples of the invention have been described and it is envisaged that improvements and modifications can take place without departing from the scope of the attached claims.

Claims

1. A process for the preparation of keratin derivatives of high molecular weight, the process including a first stage digestion step of sulfonating a keratin source by oxidative sulfitolysis followed by a second stage extraction step using controlled washing with water to thereby produce a highly S-sulfonated keratin derivative.
2. A process as recited in claim 1 in which the oxidative sulfitolysis uses cuprammonium hydroxide, or a thionate, as the oxidant, and sulfite.
3. A process as recited in claim 1 or claim 2 wherein the two stages use surfactants, heat, agitation and homogenisation to control the rate of digestion in the first stage and extraction in the second stage.
4. A process as recited in claim 2 using surfactants, heat, agitation and homogenisation to control the rate of release of residual reagents and soluble protein, thereby allowing separation of the highly S-sulfonated keratin derivative.
5. A process for the separation of a gelatinous keratin substrate from the solution of S-sulfonated keratin produced by the process as recited in any one of claims 1 to 4 wherein the S-sulfonated keratin derivative solution is treated by the use of a gentle, gravity fed filtration system followed by centrifugal separation.
6. A process as recited in any one of claims 1 to 5, that uses a combination of engineering solutions to allow continuous preparation of S-sulfonated keratin derivatives.
7. A process as recited in claim 1 wherein the solution of S-sulfonated keratin is purified using chelating agents, such as EDTA, to sequester metal ions, such as copper.
8. A process as recited in claim 1 wherein the solution containing S-sulfonated keratin derivatives is purified using ion exchange media to remove residual reagents, including metals such as copper.
9. A process as recited in claim 8 wherein the solution is concentrated prior to an ion exchange treatment through the use of ultrafiltration membranes, or similar.
10. A process as recited in claim 9 in which reagents such as copper are

isolated and reused in subsequent processes.

11. A highly S-sulfonated solution of keratin derivatives produced by the process of any one of claims 1 to 10.
12. A process for the isolation of highly S-sulfonated keratin intermediate filament proteins from the solution of keratin claimed in claim 11 wherein the highly S-sulfonated keratin intermediate filament protein is isolated by isoelectric precipitation at acidic pH.
13. A highly purified proteinaceous product produced by the process of claim 12.
14. A proteinaceous product as recited in claim 13 which is purified by the dissolution of the S-sulfonated keratin intermediate filament proteins in base in the presence of EDTA, and subsequent precipitation at acidic pH.
15. A water soluble form of S-sulfonated keratin intermediate filament proteins produced by spray drying an aqueous solution of the polymeric product as recited in claim 13.
16. A process for the production of highly S-sulfonated keratin high sulphur proteins, produced by spray drying the solution of S-sulfonated keratin derivatives purified as claimed in claims 7 and 8, and after having had the intermediate filament proteins removed by the process as claimed in claim 12.
17. A proteinaceous product produced by the process of claim 16.
18. A process for the production of soluble keratin peptides through the action of hydrogen peroxide solutions on the gelatinous residue produced by the process as recited in any one of claims 1 to 5.
19. A proteinaceous product produced by the process recited in claim 14.
20. A process for the production of soluble keratin peptides through the action of sodium sulfide solutions on the gelatinous residue produced by the process as recited in claims 1 to 5.
21. A proteinaceous product produced by the process recited in claim 20.
22. A process for the production of soluble keratin peptides through the action of proteolytic enzymes, such as those from the subtilisin, papain or trypsin families, on the gelatinous residue produced by the process as recited in claims 1 to 5.

23. The proteinaceous product produced by the process recited in claim 22.

24. A process for the treatment of copper-rich solutions produced by the process as recited in claim 2, in which wool is used as a filter media to bind copper and remove it from the solution.

25. A subsequent protein extraction process for the treatment of the copper-laden wool produced by the process recited in claim 24.

26. A method for large scale recovery of proteins from a natural source, including subjecting said natural protein source to a treatment sufficient to render at least some of the protein(s) water soluble, and subsequently separating the water soluble protein(s).

27. An installation for large scale recovery of proteins from a natural source, a treatment vessel to contain and subject a large quantity of natural protein source to a treatment sufficient to render at least some of the protein(s) contained in said feed, water soluble, and a separation apparatus to subsequently separate the water soluble protein(s).

28. An installation as claimed in claim 27 and wherein the quantity of feed is greater than 1 kg.

29. A method of selectively solubilising a protein having plurality of disulfide bonds from a mixture of proteins including subjecting said mixture of proteins to oxidative sulfitolysis to produce a soluble S-sulfonated protein fraction.

30. A method of selectively solubilising a protein as claimed in claim 29 wherein the oxidative sulfitolysis is effected in the absence of chaotropic agents.

31. A method of fractionating a mixture of proteins including the step of selectively solubilising at least one protein in said mixture by the method of claim 29 or claim 30 and removing a selectively solubilised protein.

32. A method according to any one of claims 29 to 31 wherein the mixture of proteins is naturally occurring.

33. A method according to any one of claims 29 to 32 wherein the mixture of proteins is keratin.

34. A method according to any one of claims 29 to 33 wherein the oxidative sulfitolysis is carried out by means of a discrete sulfitolysis step and a

discrete oxidation step.

35. A method according to any one of claims 29 to 34 wherein the S-sulfonated protein fraction contains at least about 4 mol% cysteine.

36. A method according to any one of claims 29 to 35 wherein the S-sulfonated protein fraction contains at least about 6 mol% cysteine.

37. A method according to any one of claims 29 to 36 wherein the S-sulfonated protein fraction contains at least about 12 mol% cysteine.

38. A method according to any one of claims 29 to 37 wherein the S-sulfonated protein fraction contains at least about 22 mol% cysteine.

39. A method for obtaining a purified protein with little or no damage to the structural integrity of the protein from an impure protein source including subjecting said protein source to a treatment sufficient to render at least some of the protein(s) water soluble, and subsequently separating the water soluble protein(s) in the absence of chaotropic agents.

40. A method according to claim 39 wherein the protein source is a naturally occurring protein source.

41. A method according to claim 39 or 40 wherein the protein source is from an animal.

42. A method according to any one of claims 39 to 41 wherein the protein source is from a plant.

43. A method according to any one of claims 39 to 42 wherein the protein source is keratin.

44. A method according to any one of claims 39 to 43 wherein the protein source is subjected to oxidative sulfitolysis.

45. A method according to any one of claims 39 to 44 wherein the water soluble protein is separated by means of filtration.

46. A method according to any one of claims 39 to 45 wherein the water soluble protein is separated by washing with water.

47. A method according to any one of claims 39 to 46 wherein the water soluble protein is separated by application of steam.

48. A method according to any one of claims 39 to 47 wherein the protein source is sequentially subject d to an oxidising agent and a sulfonating agent.

49. A method according to any one of claims 39 to 48 wherein the protein source is subjected to the sulfonating agent first, followed by the oxidising agent.

50. A method according to any one of claims 39 to 49 wherein the protein source is simultaneously subjected to an oxidising agent and a sulfonating agent.

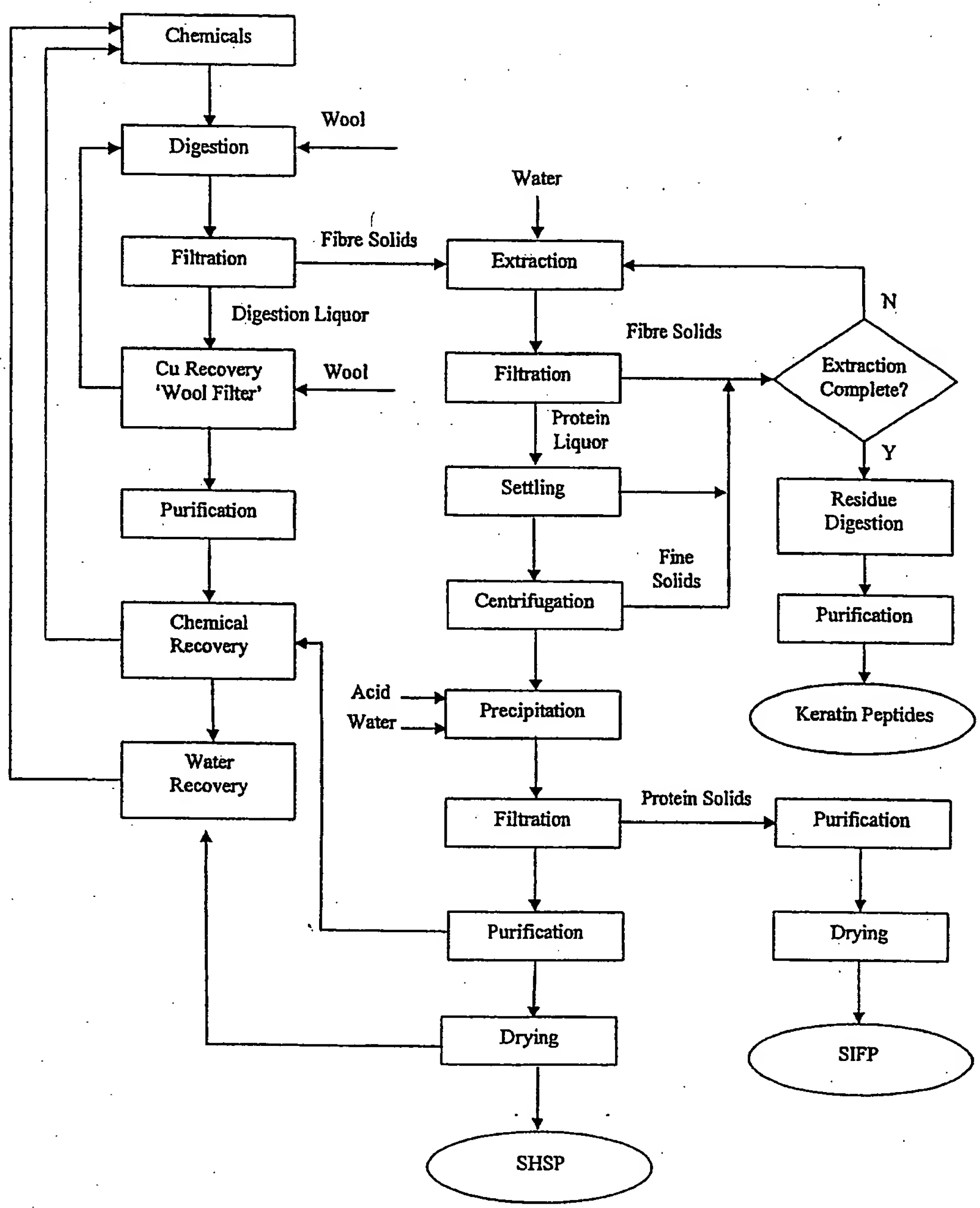

Figure 1: Protein extraction process diagram.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/NZ02/00125

A. CLASSIFICATION OF SUBJECT MATTER

Int. Cl. [7]: C07K 1/107, 1/14, 2/00.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
Database: STN. Files: CA, WPIDS, Medline. Keywords: keratin, wool, fibre, hoof, hooves, nail, hair, horn, oxid?, sulfit? sulfo?,

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	International Journal of Biological Macromolecules, vol. 8, no. 5, 1986, pages 258-264, H. Thomas et al, "In vitro reconstitution of wool intermediate filaments." See entire document, especially page 258 column 2-page 259 column 1.	1-25
X	The Biochemical Journal, vol. 92, no. 1, 1964, pages 8-18, B. S. Harrap et al, "Soluble derivatives of feather keratin." See entire document, especially page 9 column 2 - page 10 column 1.	1-25
X	Das Leder, vol. 39, no. 1, 1988, pages 1-9, H. H. Mies, "Präparative gewinnung löslicher proteine aus wolle." See entire document.	1-25

[X] Further documents are listed in the continuation of Box C   [X] See patent family annex

* Special categories of cited documents:

- "A" document defining the general state of the art which is not considered to be of particular relevance
- "E" earlier application or patent but published on or after the international filing date
- "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- "O" document referring to an oral disclosure, use, exhibition or other means
- "P" document published prior to the international filing date but later than the priority date claimed
- "T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
- "X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
- "Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
- "&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
12 September 2002	7 OCT 2002

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INTERNATIONAL SEARCH REPORT

International application No.
PCT/NZ02/00125

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	US 3567363 (L. J. Wolfram) 2 March 1971. See entire document.	1-25
A	FR 1503640 (THE GILLETTE COMPANY) 1 December 1967. See entire document.	1-25
A	WO 99/18922 (L'OREAL SOCIETE ANONYME) 22 April 1999. See entire document.	1-25
A	GB 2115427 A (L'OREAL) 7 September 1983. See entire document.	1-25
A	Australian Journal of Chemistry, vol. 14, 1960, pages 69-83, J. M. Swan, "The reaction of protein thiol and disulphide groups with cupric sulphite solutions." See entire document.	1-25
P,A	WO 02/09659 A2 (HANS SCHWARZKOPF GmbH & Co.) 7 February 2002 See entire document.	1-25

INTERNATIONAL SEARCH REPORT Information on patent family members	International application No. **PCT/NZ02/00125**

This Annex lists the known "A" publication level patent family members relating to the patent documents cited in the above-mentioned international search report. The Australian Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent Document Cited in Search Report		Patent Family Member					
US	3567363	NONE					
WO	9918922	AU	94463/98	CA	2307758	EP	1027028
		FR	2769499				
GB	2115427	DE	3305305	FR	2521571	US	4948876
WO	0209659	AU	200177546	DE	10036749		
FR	1503640	NONE					

END OF ANNEX

Box I **Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)**

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos :

   because they relate to subject matter not required to be searched by this Authority, namely:

2. ☒ Claims Nos : 26-50

   because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

   A full search was not possible on economic grounds. The invention appears to reside in the preparation of keratin derivatives, obtained via oxidative sulfitolysis followed by an aqueous extraction step. The search has therefore been limited to those claims (1-25) that are perceived to be supported by the description. Claims 26-50 are so broad in scope that a search was not economically viable.

3. ☐ Claims Nos :

   because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a)

Box II **Observations where unity of invention is lacking (Continuation of item 3 of first sheet)**

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.

☐ No protest accompanied the payment of additional search fees.